ABSTRACT

Castor (Casz1) is a zinc finger transcription factor that has been shown to be required for heart development in *Xenopus*. *Casz1* has also been linked to high blood pressure and hypertension in humans through a recent Genome Wide Association Study. The purpose of this research was to determine the role of *Casz1* in cardiac development and how *Casz1* fits into the cardiac transcription program. A mouse model was used because of the high genomic conservation between mice and humans. To determine the spatial and temporal expression of *Casz1* mRNA during murine heart development, I utilized in situ hybridization. In addition, to confirm that CASZ1 protein is present in the developing heart, I performed immunohistochemistry. My studies showed that *Casz1* is expressed in the atria and left ventricle of the developing heart. To determine the role of *Casz1* in cardiac development, I examined the cardiomyocyte mitotic index of wild type embryos and *Casz1* mutant embryos, and demonstrated that cardiomyocytes lacking *Casz1* over-proliferate. This indicates that *Casz1* regulates cardiomyocyte proliferation. Future studies are aimed at identifying genes that *Casz1* regulates, providing further insight into the cardiac gene program. These studies hold implications for understanding congenital heart defects by giving us further insight into the molecular mechanisms that regulate cardiac development.

INTRODUCTION

Congenital heart defects are the most common type of birth defect, occurring in about 1% of all live births. Congenital heart defects can take an emotional and financial toll on victims and their families, so it is important to research the mechanisms behind these defects in order to develop new therapies. Our lab is interested in the molecular mechanisms that regulate heart

development. By studying these mechanisms, we come closer to understanding how to provide treatment for congenital heart defects.

The heart is the first organ to form during development. Two groups of cells, termed the primary and secondary heart fields, contribute to the formation of the heart. Cells from the primary heart field will form a structure known as the cardiac crescent at stage E7.0. The cardiac crescent develops into the linear heart tube at stage E8.0, which then undergoes looping to become the classic 4-chambered heart around stage E9.5. Primary heart field cells give rise to the left ventricle and the two atria, whereas secondary heart field cells give rise to the right ventricle and the outflow tract[1].

Castor (*Casz1*) is a zinc finger transcription factor that had been shown to be required for heart development in *Xenopus*. The depletion of *Casz1* in Xenopus embryos causes malformation of the primitive heart tube, which inhibits proper looping of the heart tube to form the mature heart and is lethal to the embryo. *Casz1* has also been linked to high blood pressure and hypertension in humans through a recent Genome Wide Association Study (GWAS)[2]. The implications from this GWAS make it important to study the mechanisms through which *Casz1* affects cardiomyocyte differentiation and proliferation as a possible means of future treatment for heart defects. *We hypothesize that Casz1 is necessary for proper formation of structures that arise from the primary heart field.*

Casz1 is expressed in the mouse, but its role in mammalian cardiac development is poorly understood. There is high genomic conservation between mice and humans, with 87% conservation of cDNA identity and 90% conservation of protein identity, which makes the mouse a model organism for studying *Casz1*. Heart development is regulated by many different transcription factors. Our goal is to determine the role of *Casz1* during cardiac development and

how *Casz1* fits into the cardiac transcription program. An important step in doing this is to examine the effects of deleting CASZ1 in the mammalian embryo. To address this, a conditional mutation was made in mouse using CreLoxP technology. This allowed *Casz1* to be ablated at specific times in development in cells expressing *Nkx2.5,* an early cardiac marker. *Casz1* conditional knockout mice begin to show a degenerative phenotype at stage E12.5 and are embryonic lethal by stage E14.5.

My first goal was to identify the cardiac cell types and structures that express *Casz1*. To accomplish this, I examined the spatial and temporal expression of *Casz1* mRNA in the developing wild type embryo. *Casz1* mRNA is expressed in the developing heart at embryonic stage E8.0 and is dramatically decreased by stage E10.5[4]. *Casz1* mRNA was visualized by performing *in situ* hybridization on mouse embryos at stages E8.0 through E10.5 and mouse hearts at stage E13.5. The expression of *Casz1* mRNA was compared to the expression of *Nkx2.5* mRNA. *Nkx2.5* is a well-characterized cardiac-specific gene[5]. Therefore, comparing spatial and temporal expression of the *Nkx2.5* and *Casz1* genes can give insight into the expression of *Casz1*.

My studies showed that *Casz1* mRNA is expressed in the developing myocardium, particularly in the left ventricle. Through a series of antigen retrieval experiments, I demonstrated that CASZ1 protein shows similar spatial expression as mRNA. My results are consistent with the hypothesis that *Casz1* may be necessary for the proper formation of structures that arise from primary heart field cells.

Work from our lab has shown that depletion of *Casz1* in *Nkx2.5*-positive cells results in defects in the myocardial wall. These results suggest that *Casz1* has a role in regulating cardiomyocyte proliferation. To investigate this, I examined cell proliferation in *Casz1* mutant

and *Casz1* wild type mouse hearts using the proliferation marker Phosphohistone H3 (PHH3). I used the sodium-citrate based antigen retrieval method at stages E10.5 and E12.5. Cell counts were performed to determine the mitotic index in all heart cells and in cardiomyocytes. Cardiomyocytes were stained using tropomyosin (Tmy), a cardiomyocyte-specific cytosolic marker.

Through a series of antigen retrieval experiments, I demonstrated that the left and right ventricles of *Casz1* mutant embryos had increased proliferation compared to wild type embryos at stage E10.5. This is consistent with the studies done in *Xenopus*, which show an increased proliferation in *Casz1* mutant *Xenopus* embryos. The embryos at stage E12.5 do not show a statistically significant difference in proliferation between mutant ventricles and wild type ventricles, but it has been shown that there is a decrease in the total number of cardiomyocytes in the mutant ventricles at stage E12.5. This decrease is likely due to necrosis.

Interestingly, recent RNA Sequencing data has shown that genes found in secondary heart field cells are up-regulated in *Casz1* mutant embryos at stage E10.5. Because previous data showed that *Casz1* is expressed in heart structures that developed from primary heart field cells, we are currently testing the hypothesis that *Casz1* down-regulates these proteins in primary heart field cells. This down-regulation may keep the primary heart field cells from developing into secondary heart field structures. Thus, when *Casz1* is depleted, the primary heart field cells may begin to express secondary heart field proteins. Further studies will determine how *Casz1* fits into the transcriptional pathways involved in cardiomyocyte differentiation. Studying the mechanisms behind cardiac development will allow us to develop new therapies for congenital heart defects.

METHODS

In Situ Hybridization

The *in situ* hybridization probes were made of *Casz1* exons 6-7 and Nkx2.5. The probes were synthesized using T7 polymerase from BamHI linearized plasmid DNA from an adult mouse heart. The DNA was incubated at 37˚C overnight to linearize. 0.5 µl sodium dodecyl sulfate and 50 µg/ml proteinase K were added to the DNA, which was incubated at 50˚C for 30 minutes to degrade nucleases. The linearized DNA was then incubated in 5X transcription buffer, DIG RNA labeling mix, dithiothreitol, RNAse inhibitor, and RNA polymerase for 2 hours at 37˚C. 10X DNAse buffer and DNAse were added and the DNA was re-incubated at 37˚C for another 30 minutes. The DNA probes were then purified using the Qiagen RNEasy Kit and its standard procedure.

Embryos were dissected and fixed in 4% paraformaldehyde, then dehydrated with methanol and stored at -20˚C. For the whole mount *in situ*, embryos were rehydrated through 1XPBS (0.14M NaCl, 3mM KCl, 5mM Na_2HPO_4, 2mM KH_2PO_4), 0.1% tween, and methanol. Embryos were incubated in 6% hydrogen peroxide for one hour at room temperature and then in 0.56 µl/ml proteinase K for 10 minutes. The embryos were then fixed in 4% paraformaldehyde/ 0.1% glutaraldehyde for 30 minutes at room temperature. Embryos were incubated for 1 hour at 70˚C in 50% formamide, 1.3X SSC, 5mM EDTA, 0.5% CHAPS, 100 µg/mL heparin, 0.2% Tween-20, and 50 µg/mL yeast torula RNA. The embryos were then incubated in 10 µl/mL of the *Casz1* or *Nkx2.5* probe at 70˚C overnight.

The following day, the embryos were incubated for 15 minutes in 100mM maleic acid, 100mM NaCl, and 0.1% Tween-20, with a pH of 7.6. Afterwards, the embryos were incubated in 2% Boehringer Blocking Reagent/MABT for 1 hour, then incubated in 2% Boehringer

Blocking Reagent/20% heat-inactivated lamb serum/MABT for 1 hour. The embryos were then incubated in 0.05% anti-DIG antibody overnight at 4˚C. The following day, the embryos were incubated for 1 hour in 100 mM NaCl, 100 mM Tris pH9.5, 50 mM $MgCl_2$, 0.1% Tween-20, and 1.2 mg/mL Levamisol. They were then incubated in BM Purple AP Substrate in 20-minute increments until sufficient staining was observed. The embryos were then post-fixed in 4% paraformaldehyde/0.1% glutaraldehyde for 2 hours. The embryos were placed in 10XPBS (1.4M NaCl, 30mM KCl, 50mM Na_2HPO_4, 20mM KH_2PO_4), and 0.1% Tween-20 for storage. Embryos were embedded in gelatin and sectioned in 30µm sections using a vibratome.

Immunohistochemistry

Paraffin sections were done on E10.5, E12.5, and E14.5 embryos. Embryos were fixed in 4% PFA, 1XPBS overnight. They were stepped into methanol and stored in 100% methanol at 4˚C. The embryos were washed twice in ethanol for 10 minutes, then twice in 50% ethanol, 50% xylene for 10 minutes. Then the embryos were washed twice in 100% xylene for 10 minutes, once in 50% xylene, 50% paraffin for 30 minutes at 56˚C, and three times in 100% paraffin at 56˚C for 30 minutes. The tissue was then embedded in a paraffin mold and stored at 4˚C. Embryos were sectioned using a microtome at 8µm and sections were stored at 4˚C. Before antigen retrieval was performed, sections were deparaffinized and rehydrated through two 10 minute washes in 100% xylene, one 10 minute wash in 100% ethanol, one 5 minute wash in 95% ethanol, one 5 minute wash in 70% ethanol, and two 5 minute washes in ddH_2O.

Cryosectioning was performed on E7.5, E10.5, E11.5, and E12.5 embryos. The embryos were stepped into sucrose; first 15% sucrose, 1XPBS; then 30% sucrose, 1XPBS. The embryos

were then embedded in OCT and cut on a cryostat at 12μm. The sections were baked at 55˚C for 10 minutes and stored at -20˚C.

Antigen retrieval of both frozen and paraffin sections was performed using a 10mM sodium citrate buffer (pH 6.0) to break protein crosslinks. Sections were blocked in 10% heat-inactivated calf serum, 1% TritonX-100, 1XPBS for 1 hour in a humid chamber, then incubated in primary antibody in 1% Heat-inactivated calf serum, 0.1% TritonX-100, 1XPBS overnight at 4˚C. Slides were incubated in secondary antibody in 1% Heat-inactivated calf serum, 0.1% TritonX-100, 1XPBS for 1 hour at room temperature. Sections were stained with DAPI in ethanol for 10 minutes.

The primary antibodies used were:

Castor (Casz1):

Casz1 e14 (Santa Cruz) SC-135453 rabbit IgG (1:1000)

Tropomyosin (Tmy):

CH1 (DSHB) mouse monoclonal IgG_1 (1:50 concentration)

Phosphohistone H3

PHH3 millipore 06-570 rabbit (1:200 concentration)

The secondary antibodies used were:

Donkey-anti-rabbit Alexa 488 (green) (1:1000 concentration)

Goat-anti-mouse Alexa 546 (red) (1:1000 concentration)

Cell Counting

All pictures were taken using a Zeiss 700 confocal microscope. For proliferation studies, cell counts were performed using the aid of Photoshop counting software. For each stage,

antibody staining was performed on anterior, mid, and posterior sections of both a *Casz1* null embryonic mouse heart and wild type embryonic mouse heart. For each section, two pictures were taken of each of the following; the left atria, left ventricle, right atria, and right ventricle. Cell counts were taken for the total number of cells, the number of proliferating cells, the total number of cardiomyocytes, and the number of proliferating cardiomyocytes. The mitotic index was calculated by dividing the number of proliferating cells by the number of overall cells. All calculations were done using the Microsoft Office Excel software.

RESULTS

Casz1 mRNA is Expressed in the Primitive Left Ventricle and Atrial Areas in the Primitive Heart Tube (E8.0)

In situ hybridization was performed on E8.0 mouse embryos using a *Casz1* exon 6-7 probe to determine *Casz1* expression in the primitive heart tube. Transverse sections imaged using an Olympus IX81 microscope show that *Casz1* is expressed throughout the primitive heart tube. Specifically, *Casz1* expression in the primitive heart tube is strongest in the primitive left ventricle and primitive atrial sections (Fig.1 C-G), as well as in the bulbus cordis (Fig.1 A,B) and sinus venosus (Fig.1 H,I). *Casz1* expression was found in myocardial tissue, which makes up the outside of the primitive heart tube and the bulbus cordis

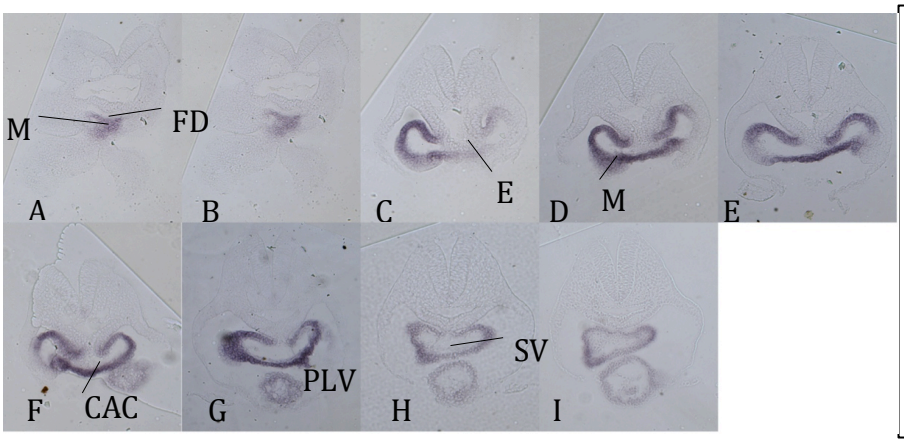


Figure 1. Casz1 mRNA is expressed in the primitive heart tube. (A-I) Transverse vibratome sections of E8.0 mouse embryo using a Casz1 exon 6-7 probe; (MT) Mesodermal tissue (FD) Foregut Diverticulum (EC) Endothelial cells lining the primitive heart tube (MC) Myocardial tissue (CAC) Common atrial chamber (PLV) primitive left ventricle (SV) Sinus venosus.

Casz1 mRNA is Expressed in the Common Ventricular Chamber and Common Atrial Chamber in the Looped Heart (E9.5)

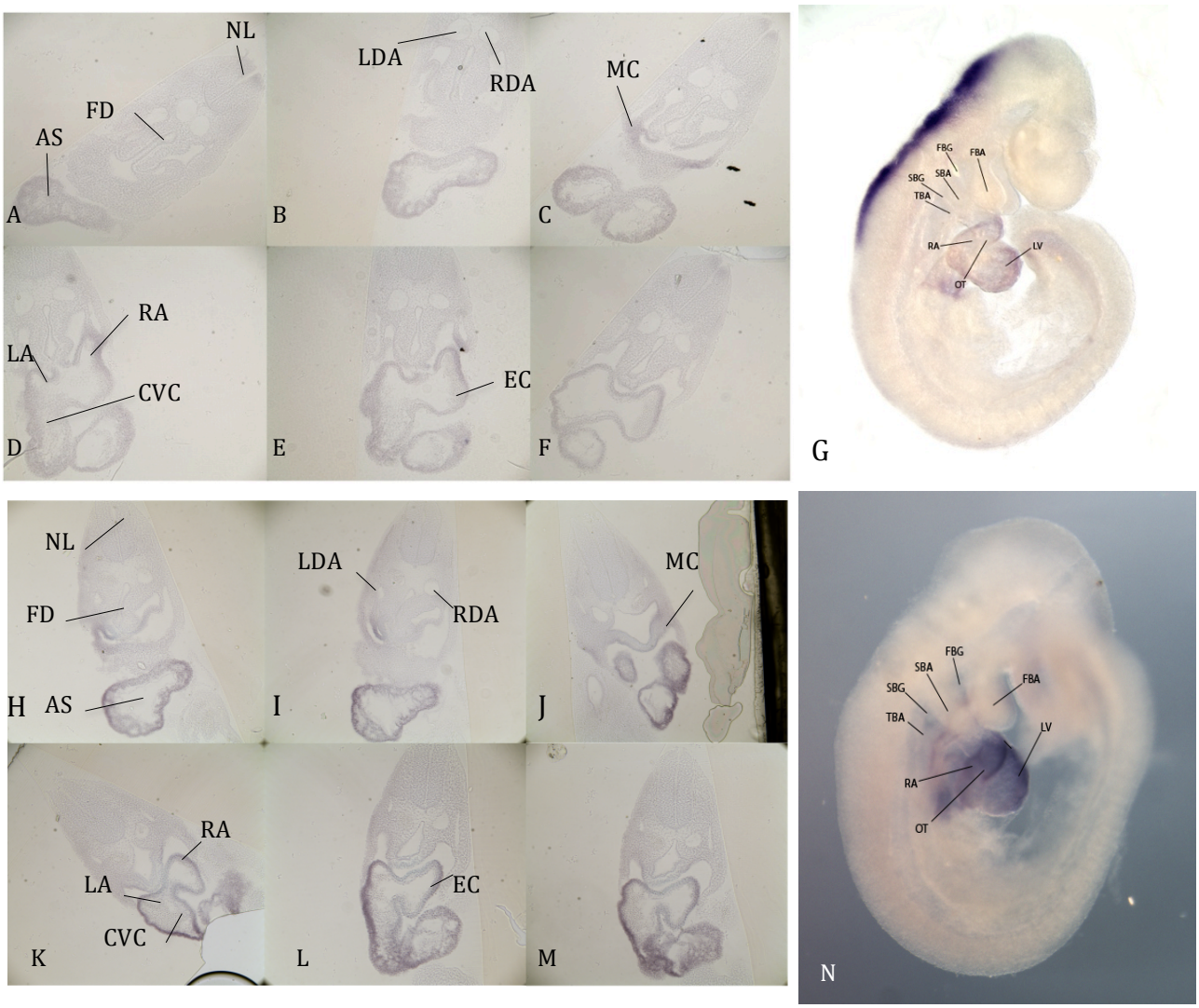


Figure 2. Casz1 and Nkx2.5 are expressed in the looped heart at stage E9.5. (A-F) Transverse vibratome sections of E9.5 mouse embryo using a Casz1 exon 6-7 probe. (H-M) Transverse vibratome sections of E9.5 embryo using an Nkx2.5 probe. (FD) Foregut diverticulum (NL) Neural lumen (AS) Aortic Sac (RDA) Right dorsal aorta (LDA) left dorsal aorta (MC) Myocardial tissue. (RA) Right component of common atrial chamber (LA) Left component of common atrial chamber (CVC) Common ventricular chamber of heart (EC) Endocardial tissue (G) Whole mount E9.5 mouse embryo using a Casz1 exon 6-7 probe. (N) Whole mount E9.5 mouse embryo using an Nkx2.5 probe. (FBA) First brachial arch (FBG) First brachial groove (SBA) Second brachial arch (SBG) Second brachial groove (TBA) Third brachial arch (TBG) Third brachial groove (RA) Right atria (LV) Left ventricle (OT) Outflow tract

In situ hybridization was performed on E9.5 mouse embryos using a *Casz1* exon 6-7 probe and an *Nkx2.5* probe to determine *Casz1* and *Nkx2.5* mRNA expression in the looped heart. Transverse sections of an embryo stained for *Casz1* (Fig.2 A-F) show that *Casz1* is

expressed in the aortic sac (Fig.2 A,B), the left and right sides of the common atrial chamber (Fig.2 D-F), and the common ventricular chamber of the heart (Fig.2 D-F). *Casz1* was also expressed in the neural tissue of the neural lumen (Fig.2A).

Transverse sections of an *Nkx2.5* embryo (Fig.2 H-M) show that *Nkx2. 5* is expressed in the aortic sac (Fig.2 H,I), the left and right sides of the common atrial chamber (Fig.2 K-M), and the common ventricular chamber of the heart (Fig.3 K-M). *Nkx2.5* expression was strongest in the myocardial tissue. *Nkx2.5* expression was not observed in neural tissue (Fig.2 A).

Whole mount images of E9.5 mouse embryos show expression of both *Casz1* and *Nkx2.5* mRNA in the right side of the common atrial chamber, the left ventricle, and the outflow tract. *Casz1* is expressed in the neural tube (Figure 2 g). *Nkx2.5* is expressed in the first, second, and third brachial arches (Figure 2 n).

Casz1 mRNA is Expressed in the Common Atrial Chamber and Left Ventricle in the Looped Heart (E10.5)

In situ hybridization was performed on E10.5 mouse embryos using a *Casz1* exon 6-7 probe and an *Nkx2.5* probe to determine *Casz1* and *Nkx2.5* expression at a later stage in the looped heart. Whole mount images of an E10.5 *Nkx2.5* embryo (Fig.3 A,B) show expression of *Nkx2.5* in the left and right components of the common atrial

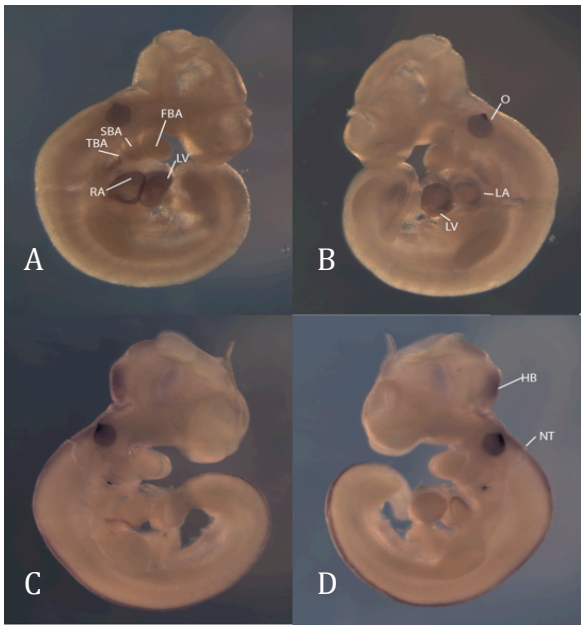


Figure 3. *Casz1* mRNA is expressed in the looped heart at stage E10.5. (A,B) Whole mount E10.5 mouse embryo using an *Nkx2.5* probe. (C,D) Whole mount E10.5 mouse embryo using a *Casz1* exon 6-7 probe. (FBA) First brachial arch. (SBA) Second brachial arch (TBA) Third brachial arch (RA) Right component of common atrial chamber (LV) Left ventricle (O) Otocyst (LA) Left component of common atrial chamber (NT) Neural tube (HB) Hindbrain.

chamber and in the left ventricle. Whole mount images of an E10.5 *Casz1* embryo (Fig.3 C,D) show expression of *Casz1* in the left side of the common atrial chamber and the left ventricle. *Casz1* is expressed in the neural tube and hindbrain at this stage. Both *Nkx2.5* and *Casz1* were expressed in the otocyst of the E10.5 embryos. This is unprecedented expression of both *Nkx2.5* and *Casz1*, and is likely due to dye trapping in the embryo.

Casz1 and Nkx2.5 mRNA is Expressed in the Left Ventricle of the Looped Heart (E13.5)

In situ hybridization was performed on E13.5 mouse hearts using a *Casz1* exon 6-7 probe and an *Nkx2.5* probe to determine *Casz1* and *Nkx2.5* mRNA expression at a later stage in the looped heart. *Nkx2.5* is expressed in the outer curvature of both the left and right ventricles. Expression of *Nkx2.5* is also seen in the left and right atria and in the interventricular septum (Fig.4 A,B). *Casz1* is expressed in the outer curvature of the left ventricle, the interventricular septum, and the bulbar cushion tissue. *Casz1* also is lightly expressed in the left and right atria (Fig.4 C,D).

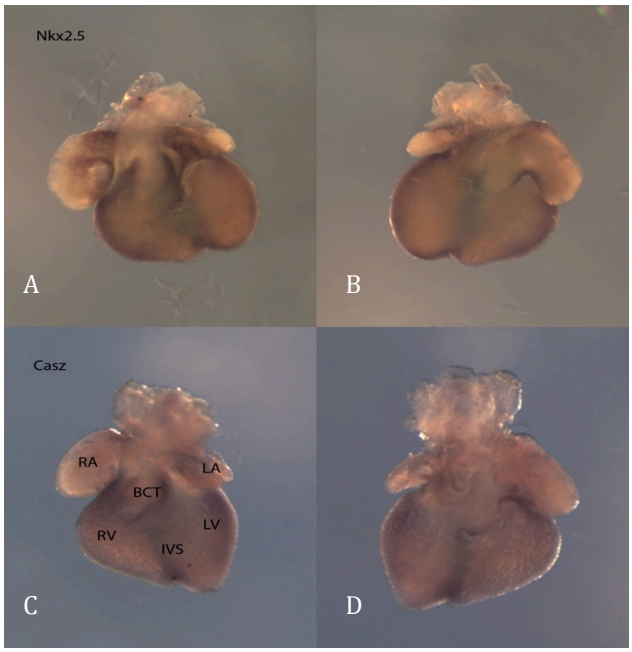


Figure 4. *Casz1* and *Nkx2.5* are expressed in the looped heart at stage E13.5. (A,B) Image of whole E13.5 mouse heart using an *Nkx2.5* probe. (C,D) Image of whole E13.5 mouse heart using a *Casz1* exon 6-7 probe. (RA) Right atria (LA) Left atria (BCT) Bulbar cushion tissue (RV) Right ventricle (LV) Left ventricle (IVS) Interventricular septum

CASZ1 Protein is Expressed in the Aortic Sac and the Left Side of the Common Ventricular Chamber

Confocal imaging showed nuclear staining of CASZ1 protein in cardiomyocytes. Nuclear staining was shown in both the aortic sac (Fig.5 A-C) and common ventricular chamber (Fig.5 D-F). CASZ1 staining was highly specific to the cardiomyocytes and was present in almost all cardiomyocytes photographed in these sections. Both the aortic sac and common ventricular chamber showed similar amounts of staining.

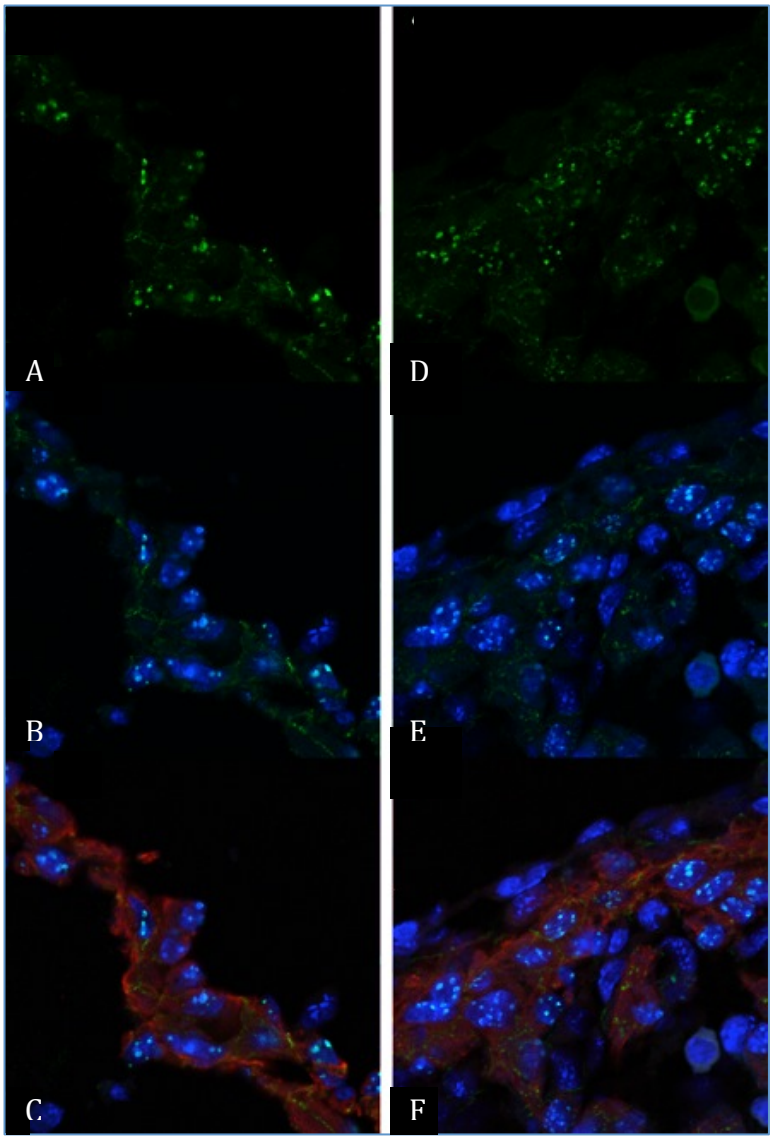


Figure 5. CASZ1 is expressed in cardiomyocytes of E10.5 embryos. Transverse cryosections of E10.5 embryos stained with CASZ1 antibody (A,D); CASZ1 antibody and DAPI (B,E); and CASZ1 antibody, CH1 antibody, and DAPI (C,F). Photographs were taken of the aortic sac (A-C) and the common ventricular chamber (D-F). Colors indicate CASZ1 (green), Tmy (red), DAPI (blue)

Casz1 Mutant Hearts Have a Higher Mitotic Index at Stage E10.5 Than Wild Type Hearts

Representative sections of the left ventricles from *Casz1* null and wild type hearts at stage E10.5 are shown in Figure 6. PHH3 nuclear staining is seen in each section. The cardiomyocyte mitotic index was calculated for each mouse using the cardiomyocyte proliferation data obtained for each individual section. At stage E10.5, the *Casz1* null hearts have an average cardiomyocyte mitotic index of 0.2568, compared to a mitotic index of 0.1764 in the wild type cardiomyocytes.

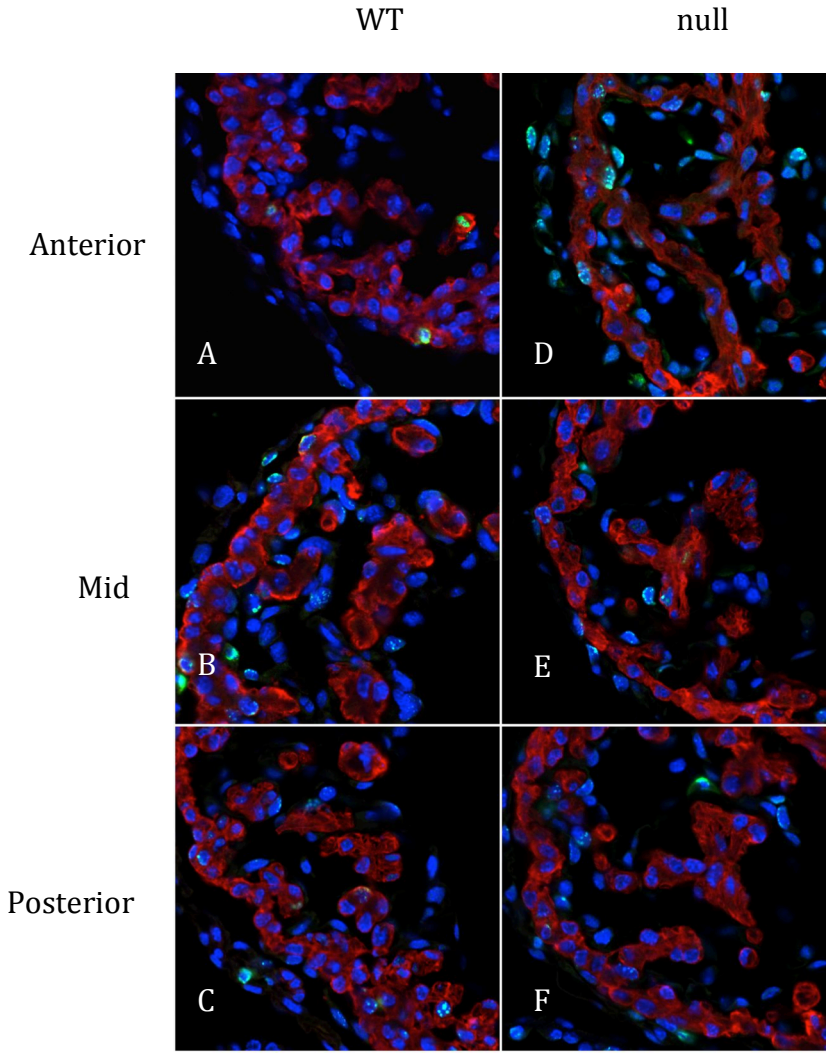


Figure 6. PHH3 is expressed in E10.5 wild type and *Casz1* null hearts. Transverse paraffin sections of E10.5 embryos stained with PHH3 antibody (green), TMY antibody (red) and DAPI (blue). Photographs were taken of the Left ventricles of wild type anterior (A), middle (B), and posterior (C) heart sections; and left ventricles of *Casz1* null anterior (D), middle (E), and posterior (F) heart sections.

Casz1 Mutant and Wild Type Hearts have Similar Mitotic Indexes at Stage E12.5

Representative sections of the left ventricles from the *Casz1* null and wild type hearts at stage E12.5 are shown in Figure 7. PHH3 nuclear staining is seen in each section. The cardiomyocyte mitotic index was calculated for each mouse using the cardiomyocyte proliferation data obtained for each individual section. At stage E12.5, the *Casz1* null hearts have an average cardiomyocyte mitotic index of 0.3326, compared to a mitotic index of 0.3343 in the wild type cardiomyocytes.

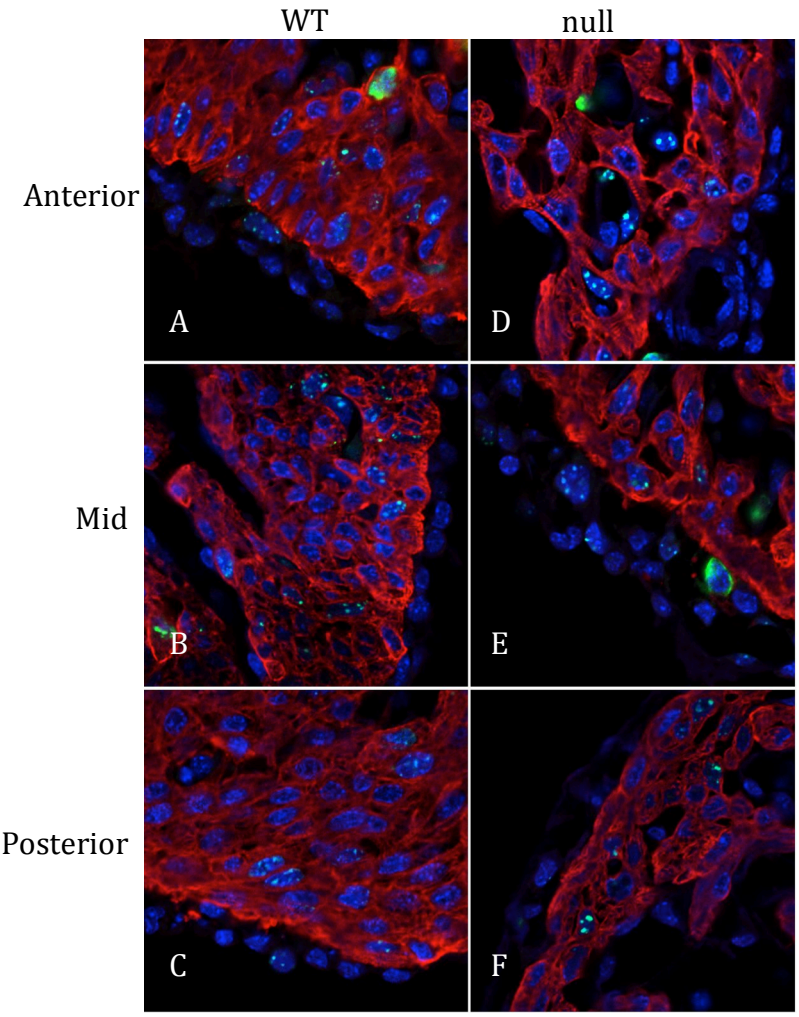
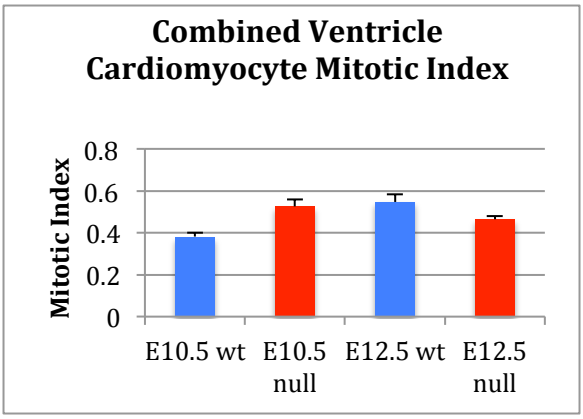


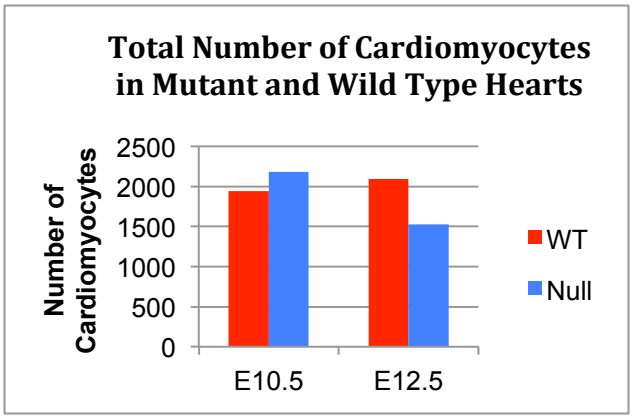
Figure 7. PHH3 is expressed in E12.5 wild type and *Casz1* null hearts. Transverse paraffin sections of E12.5 embryos stained with PHH3 antibody (green), TMY antibody (red) and DAPI (blue). Photographs were taken of the Left ventricles of wild type anterior (A), middle (B), and posterior (C) heart sections; and left ventricles of *Casz1* null anterior (D), middle (E), and posterior (F) heart sections.

There is Increased Cardiomyocyte Proliferation in the Ventricles of Casz1 Mutant Mice at E10.5

There is a statistically significant difference in proliferation in the ventricles of wild type hearts compared to mutant hearts at stage E10.5. The left and right ventricles of the wild type heart at E10.5 had a combined mitotic index of 0.381, whereas the ventricles of the mutant heart at this stage had a mitotic index of 0.526. The ventricles of the E12.5 hearts did not show a statistically significant difference in mitotic indexes between the wild type and mutant embryos (Graph 1). It is important to note, however, that despite the increase in proliferation in mutant ventricles at stage E10.5, there is a decrease in the overall number of cardiomyocytes in the ventricles of the mutant heart at stage E12.5 (Graph 2).



Graph 1. Ventricles of mutant hearts have higher mitotic index at stage E10.5. Combined mitotic indexes of the left and right ventricles in mutant hearts versus wild type hearts at each stage.



Graph 2. Decrease in number of cardiomyocytes in mutant heart at E12.5. The number of cardiomyocytes in the ventricles of mutant and wild type embryos at stages E10.5 and E12.5.

DISCUSSION

By performing *in situ* hybridization, it was possible to distinguish the types of tissue in which *Casz1* is regularly expressed, as well as the structures in which it is expressed. *Casz1* expression was seen most notably in myocardial tissue. Myocardial tissue makes up the outer

components of the atria and ventricles, as well as their primitive counterparts. This expression of *Casz1* in the atria and left ventricle is in accordance with the hypothesis that cardiac progenitor cells lacking *Casz1* cannot contribute to structural descendants of the primary heart field. The expression of *Casz1* in the neural tissue was in accordance with existing data on the expression of *Casz1*[2,3,4].

The decrease in *Casz1* mRNA expression in the heart at stage E10.5 corresponds to previous evidence that *Casz1* is important for the looping of the primitive heart tube and the formation of the heart chambers, both of which occur before stage E10.5[1,4]. This temporally linked expression suggests that the role *Casz1* plays in cell development is carried on without the transcription of new *Casz1* mRNA at stage E10.5. This is concurrent with the hypothesis that *Casz1* is required for early cardiac cell fate decisions.

By stage E13.5, all four chambers of the mouse heart have formed, but are not yet fully developed. In E13.5 mouse hearts, *Casz1* mRNA is once again expressed in the outer curvature of the left ventricle. This is in accordance with the hypothesis that *Casz1* contributes to the formation of the left ventricle. *Casz1* is also expressed in the interventricular septum and the bulbar cushion tissue, which will form the bulbar septum. This suggests that *Casz1* may function in the development of this septal tissue. The increased expression of *Casz1* in the E13.5 embryo compared to the E10.5 embryo suggests that *Casz1* functions in the maturation of the looped heart.

Experimental data was collected from *in situ* hybridizations performed using mouse embryos at stages E8.0 to E10.5 and mouse hearts at stage E13.5. This data supports the hypothesis that *Casz1* is necessary for cardiac progenitor cells to contribute to the structures that

arise from primary heart field cells. In order to test this hypothesis further, I used immunohistochemistry to look at the expression of CASZ1 protein.

Immunohistochemistry experiments performed using E10.5 mouse embryos showed nuclear expression of CASZ1 in most cardiomyocytes in the common ventricular chamber and aortic sac. The spatial expression of CASZ1 protein corresponds to the expression of *Casz1* mRNA that was observed through the *in situ* hybridization experiments. It would be beneficial in the future to repeat this experiment and to determine the differences between those cardiomyocytes that express CASZ1 in the E10.5 embryo and those that do not, to see if there is any significance in this spatial dissonance. It is possible that differences in the identities of the cardiomyocyte progenitor cells play a role in this distinction. To further characterize CASZ1, proliferation studies were done in mutant and wildtype mice.

In *Xenopus*, it has been shown that ablation of *Casz1* results in increased proliferation at the ventral midline, but the role of *Casz1* in mammalian cardiomyocyte proliferation is unknown[2]. Using PHH3 staining in mouse embryos, it has now been shown that mice at embryonic stage E10.5 also display increased proliferation in *Casz1* null cardiomyocytes compared to wild type cardiomyocytes

Stage E10.5 mice showed a significant increase in cardiomyocyte proliferation in the ventricles of *Casz1* null embryos compared to those of wild type embryos. Stage E12.5 mice showed no significant difference in proliferation between the two conditions. Interestingly, there was a decrease in the number of cardiomyocytes in the ventricles of *Casz1* mutant embryos at stage E12.5 compared to E10.5, despite the increase in proliferation seen at stage E10.5. This is likely due to necrosis, as it has been shown that *Casz1* mutant embryos begin to show a degenerative phenotype around stage E12.5. My proliferation results show that *Casz1* is

required for cardiac development. I have also shown that *Casz1* is another transcription factor that we can add to the mammalian cardiac program.

From the data obtained, it appears that *Casz1* is primarily associated with the left ventricle and the atria. Recent RNA sequencing data showed that secondary heart field proteins are up-regulated in *Casz1* mutant embryos at stage E10.5. We believe that *Casz1* may be responsible for keeping secondary heart field proteins from being aberrantly expressed in primary heart field cells.

Further studies are currently being done to determine if these secondary heart field proteins and CASZ1 are expressed in mutually exclusive cardiomyocytes. We believe the increase in the amount of these proteins may occur because they are being improperly expressed in primary heart field cells. It is therefore necessary to determine if these secondary heart field proteins are being up-regulated specifically in primary heart field cells when *Casz1* is removed.

In the future, it will be important to determine which genes are specifically being targeted by *Casz1*, and which genes are downstream targets of the molecular pathway. My studies on cardiomyocyte proliferation take us one step closer to decoding the molecular mechanisms behind heart development. These mechanisms will be important in the future as we develop new therapies for congenital heart defects.

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